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RESOLUTION OF FUNGAL CELLULASE BY ZONE ELECTROPHORESIS

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Results of paper and column chromatography (1, 2) and paper electrophoresis (3) of crude preparations of fungal cellulase have pointed toward the multiple nature of this system. Results of ultracentrifugation and moving boundary electrophoresis of fractionated cellulase have, on the other hand, suggested its unienzymatic nature (4). In the present study, zone electrophoresis over extended distances has been utilized to provide more critical data bearing on this question. The findings support the hypothesis of a multiple enzyme system.

Materials and Methods

Enzyme Preparations—Cellulase solutions were prepared in about 25 liter lots by growing *Myrothecium verrucaria* QM 460 on a 0.4 per cent suspension of filter paper in 12 gallon carboys at room temperature for 2 weeks. The medium used was that of Reese, Siu, and Levinson (5). Aeration was provided at the rate of about 25 liters per minute. Special precautions were taken to avoid contamination. Changes in protein, activity, and pH in filtrates of a typical culture during the incubation are shown in Fig. 1. It will be noted that the production of protein was parallel with that of activity. The unit of activity is defined later.

Following filtration through cheese-cloth and clarification in a Sharples centrifuge, the enzyme-containing medium was concentrated at 5° in a pressure ultrafilter (6), dialyzed in collodion bags against distilled water until freed of salts, and lyophilized to yield the crude enzyme preparations used for electrophoretic studies. The final recovery of enzyme activity approached 80 per cent. On the average, the preparations contained equal amounts of protein and carbohydrate, and the activity was about 24 units per mg. of total solids. The carbohydrate was found by chromatographic tests to contain mannose and galactose but no glucose.[1] Its origin is unknown.

Zone Electrophoresis—Zone electrophoresis was carried out with potato

[1] The chromatographic tests were made by Dr. Elwyn T. Reese and Mr. William Gilligan.

starch[2] as the supporting medium in an apparatus similar to that of Kunkel and Slater (7). The starch block was favored over paper electrophoresis because of the smaller likelihood of adsorption and of hydrolytic action of the enzyme on the starch. pH changes at the electrodes were avoided by circulating the contents of the outside electrode vessels with the aid of a pump,[3] the fluid being drawn from the cathode vessel into the anode vessel and then allowed to return by way of a siphon. To prevent accumulation in the siphon of gas from the electrodes, the siphon was mounted as an inverted V, at the top of which a tube was attached for collecting the gas.

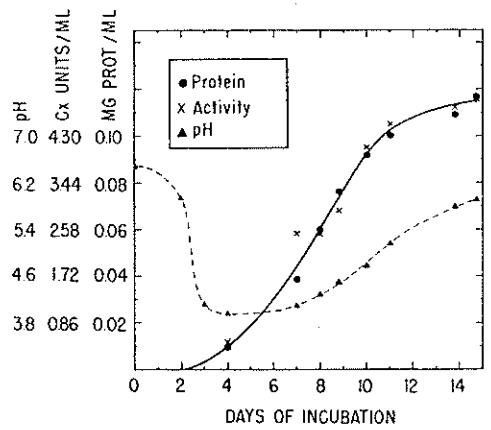

FIG. 1. Production of protein and cellulase and changes in pH in filtrates of *M. verrucaria* growing on cellulose.

The runs were made at 5° in buffers of 0.05 ionic strength, principally in sodium phosphate at pH 7. The starting sample, amounting to 100 mg. of crude enzyme in 2 ml. of buffer, was introduced into a 4 cm. slit cut crosswise in a 50 × 10 × 1.3 cm. block of the starch. The slit was located near the anode end of the block to compensate for the electroosmotic flow of buffer solution toward the cathode.

Quantitative recovery was sacrificed for improved resolution of the components by discarding strips 2 cm. wide from each of the long sides of the block at the completion of each run. This procedure reduced the overlapping that results from the observed curvature of the zones of the components. The remaining (center) strip of the block was then cut crosswise into sections 1 cm. wide, each of which was extracted with 20 ml. of phosphate buffer, 0.05 ionic, pH 7. Finally, analyses for protein, carbohydrate, and activity were made on suitable aliquots of the extracts.

[2] Chromatographic potato starch sold by the Amend Drug and Chemical Company, Inc., New York, was found to be more satisfactory than certain other commercial preparations tested because of its low content of soluble reducing sugar. The starch was not washed before use.

[3] A pump sold by Sigmamotor, Inc., Middleport, New York, was found to be particularly useful for this purpose, since it avoided direct contact of the fluid being pumped with the pump parts.

Analyses[4]—Protein concentrations were determined by the method of Lowry, Rosebrough, Farr, and Randall (8). Bovine plasma albumin was employed as a standard. The results agreed with those obtained by the micro-Kjeldahl method (9).

Carbohydrate analyses were carried out by the method of Rimington (10), with use of glucose as the standard.

Enzyme activities were determined as "Cx units" by the method of Reese and coworkers (1, 2, 5). The term Cx is used (5) to designate the particular enzyme system which hydrolyzes the 1,4-β-glucosidic linkage as found in cellulose and as measured by the amount of reducing sugars obtained by enzymatic hydrolysis of carboxymethyl cellulose. The Cx unit is defined (2) as the amount of enzyme in 10 ml. of assay medium (0.5 per cent Hercules carboxymethyl cellulose No. 50T in 0.05 M citrate at pH 5.4) required to give a reducing value as glucose of 0.40 mg. per ml. in an hour at 50°.

Results

Electrophoretic patterns of individual preparations of crude enzyme were found to be comparable in the number and relative positions of observed active components. It appeared justified, therefore, to pool all of the preparations in order to have available a larger, more completely uniform stock of the enzyme.

Results obtained with the pooled enzyme, when this was subjected to electrophoresis in phosphate buffer at pH 7 for 42 hours at 400 volts, are presented in Fig. 2. The pattern of distribution of enzyme activity indicates the presence of at least eight distinct enzyme components, one of which was completely separated from the others. The pattern was closely confirmed in a duplicate experiment. In control runs with plasma albumin and hemoglobin, it was found that migration under the conditions used with the enzyme was normal and undisturbed and, further, that complete resolution of the albumin and hemoglobin was readily obtained in tests with mixtures of the two proteins.

It appeared that a more complete resolution of the enzyme components should be possible if the time of electrophoresis were extended still further. This was accomplished, without lengthening the block, by making a run similar to the above, removing the anode section of the block up to and

[4] Mr. Alfred Sunseri assisted with the analyses.

including Component I, shifting the remaining cathode end of the block toward the anode, and attaching a fresh starch block to the incomplete cathode end. After electrophoresis was continued for an additional 40 hours at 400 volts, the distribution of components was that shown in Fig.

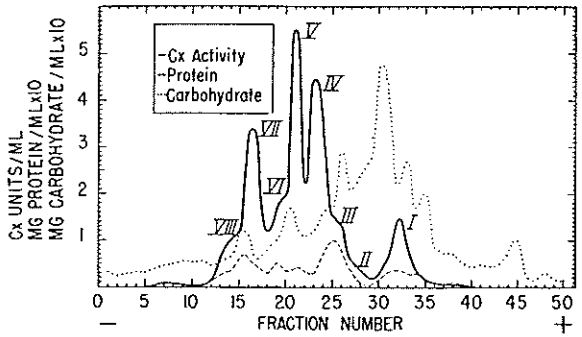

FIG. 2. Distribution of protein, carbohydrate, and activity in zone electrophoresis of crude cellulase at pH 7 for 42 hours at 400 volts.

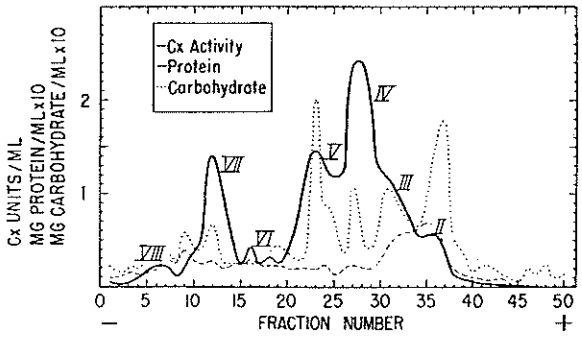

FIG. 3. Distribution of protein, carbohydrate, and activity in zone electrophoresis of crude cellulase at pH 7 for 40 hours at 400 volts, following preliminary electrophoresis at pH 7 for 42 hours at 400 volts and removal of Component I contained in anode end of block.

3. Here it may be noted that Components VII and VIII have now been practically completely separated from the others. The presence of Component I in the anode section removed earlier in the run is shown by the distribution pattern of Fig. 4.

Further electrophoretic studies showed that the resolution of the components was not improved when tested in Veronal and in glycine-NaOH buffers at pH levels up to 10, and that it was much poorer when tested in acetate and in glycine-HCl buffers at pH levels down to 3. The distribution of protein and carbohydrate at pH 3 (Fig. 5) was of particular significance, however, in that it demonstrated that only a fraction of the total protein in these preparations, and probably none of the carbohydrate, was associated with cellulase activity.

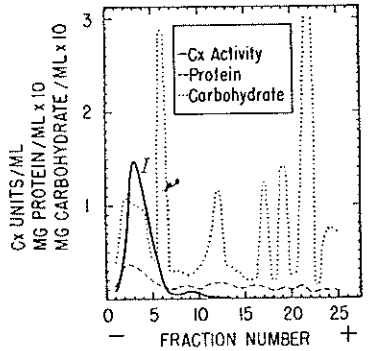

FIG. 4. Distribution of protein, carbohydrate, and activity in anode end of block after zone electrophoresis at pH 7 for 40 hours at 400 volts.

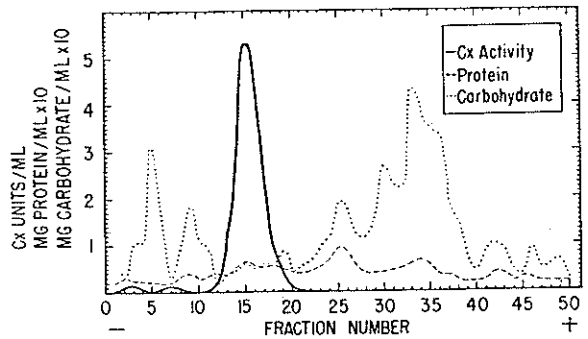

FIG. 5. Distribution of protein, carbohydrate, and activity in zone electrophoresis of crude cellulase at pH 3 for 12 hours at 400 volts.

The total recoveries of protein, carbohydrate, and activity in typical runs at different pH values are presented in the first part of Table I. Recoveries in controls, in which various mixtures of enzyme, buffer, and starch were gently agitated overnight without being subjected to electrophoresis, are also shown. The proportions of the enzyme, buffer, and starch in the controls were the same as those used in the extraction of the starch block sections. It may be seen from Table I that in the electrophoresis runs the recoveries of protein and activity were somewhat low, while those of carbohydrate were quite high.

The possibility of loss of protein and activity due to adsorption on the

starch appears to be ruled out by the fact that in the controls the recoveries of these factors were not lower in the presence of starch than in its absence. The losses were, therefore, undoubtedly largely the result of discarding the 2 cm. strips from the sides of the blocks, as mentioned earlier, although in the runs at pH 3 the apparent loss in protein is partially attributable to an effect of glycine buffer of pH 3 on the analyses. The greater losses in activity in runs at pH 3 and pH 10 than at pH 7 may be attributed to inactivation, as demonstrated by the controls and as would be expected from previous work (11, 12). The fact that at pH 7 the recovery of activity

TABLE I

Per Cent Recovery of Protein, Carbohydrate, and Activity in Electrophoresis Runs and in Controls

Description of test	Protein	Carbohydrate	Activity
Electrophoresis run, pH 3	74	160	25
" " " 7	79	152	38
" " " 10	80	153	27
Control, enzyme + buffer + starch, pH 3	60	223	65
" " + " + " " 7	103	269	109
" " + " + " " 10	97	171	71
" " + " pH 3	70	91	77
" " + " " 7	90	108	94
" " + " " 10	91	97	9
" buffer + starch, pH 3		14*	
" " + " " 7		24*	
" " + " " 10		18*	

* These values are calculated on the basis of the amount of carbohydrate which would have been contributed by the enzyme used in the other controls had the enzyme been present without acting on the starch.

was lower than that of protein may be explained by a synergistic effect (2), whereby the different enzymes can show less activity when separated from one another than when in mixtures.

The results of the controls of starch plus buffer show that the high carbohydrate values obtained in the electrophoresis runs could have been only partially due to the presence of soluble reducing sugars in the starch used as the supporting medium. It may be concluded, therefore, that the high carbohydrate values must have been due primarily to the presence of amylase in the preparations.

DISCUSSION

The observed separation of active enzyme components does not appear to be the result of disturbances such as convection during electrophoresis,

since the conditions were found to be entirely suitable when known proteins were tested. The findings lend strong support, therefore, to the hypothesis of the multiple nature of the cellulase system.

The most probable reason for the reported failure to demonstrate multiple components by moving boundary electrophoresis (4) is the use of too short distances of migration. The spread of the enzyme reported with the moving boundary method was only about one-fortieth that attained with the starch block. Less likely is the possibility that the single component resulted from a difference in conditions under which the enzyme was elaborated by the fungus. Also less likely is the possibility of fractionation of the enzyme during the purification steps, since there appears to be no reason to expect that the particular precipitating agents used should succeed in effecting a sharp separation of closely related enzyme proteins.

Final proof for the multiplicity of enzymes in the cellulase system must await their complete separation from one another and from inactive contaminants. The use of zone electrophoresis, particularly preparative designs (13–15), is clearly indicated for this purpose, although it will probably need to be supplemented by chromatographic (2, 3, 16), precipitation (4), and other methods. Comparison of the isolated enzyme components with respect to activity per unit weight, substrate specificity, physical properties, amino acid composition, and other properties will be of particular interest and importance.

SUMMARY

Zone electrophoresis of crude fungal cellulase over extended distances has indicated the multiple nature of the enzyme system.

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